Genetic Variation in the *PNPLA3* Gene Is Associated with Alcoholic Liver Injury in Caucasians

Felix Stickel,[1]* Stephan Buch,[2]* Katharina Lau,[3] Henriette Meyer zu Schwabedissen,[4] Thomas Berg,[5] Monika Ridinger,[6] Marcella Rietschel,[7] Clemens Schafmayer,[8] Felix Braun,[8] Holger Hinrichsen,[2] Rainer Günther,[2] Alexander Arlt,[2] Marcus Seeger,[2] Sebastian Müller,[9] Helmut Karl Seitz,[9] Michael Soyka,[10] Markus Lerch,[11] Frank Lammert,[12] Christoph Sarrazin,[13] Ralf Kubitz,[14] Dieter Häussinger,[14] Claus Hellerbrand,[15] Dieter Bröring,[8] Stefan Schreiber,[2] Falk Kiefer,[7] Rainer Spanagel,[7] Karl Mann,[7] Christian Datz,[16] Michael Krawczak,[17] Norbert Wodarz,[6] Henry Völzke,[3] and Jochen Hampe[2]

A recent genome-wide study revealed an association between variation in the *PNPLA3* gene and liver fat content. In addition, the *PNPLA3* single-nucleotide polymorphism rs738409 (M148I) was reported to be associated with advanced alcoholic liver disease in alcohol-dependent individuals of Mestizo descent. We therefore evaluated the impact of rs738409 on the manifestation of alcoholic liver disease in two independent German cohorts. Genotype and allele frequencies of rs738409 (M148I) were determined in 1,043 alcoholic patients with or without alcoholic liver injury and in 376 at-risk drinkers from a population-based cohort. Relative to alcoholic patients without liver damage (n = 439), rs738409 genotype GG was strongly overrepresented in patients with alcoholic liver cirrhosis (n = 210; OR 2.79; $P_{genotype} = 1.2 \times 10^{-5}$; $P_{allelic} = 1.6 \times 10^{-6}$) and in alcoholic patients without cirrhosis but with elevated alanine aminotransferase levels (n = 219; OR 2.33; $P_{genotype} = 0.0085$; $P_{allelic} = 0.0042$). The latter, biochemically defined association was confirmed in an independent population-based cohort of at-risk drinkers with a median alcohol intake of 300 g/week (OR 4.75; $P_{genotype} = 0.040$; $P_{allelic} = 0.022$), and for aspartate aminotransferase (AST) levels. Frequencies of allele *PNPLA3* rs738409(G) in individuals with steatosis and normal alanine aminotransferase (ALT) and AST levels were lower than in alcoholics without steatosis and normal ALT/AST ($P_{combined} = 0.03$). The population attributable risk of cirrhosis in alcoholic carriers of allele *PNPLA3* rs738409(G) was estimated at 26.6%. *Conclusion:* Genotype *PNPLA3* rs738409(GG) is associated with alcoholic liver cirrhosis and elevated aminotransferase levels in alcoholic Caucasians. (HEPATOLOGY 2011;53:86-95)

Alcoholic liver disease (ALD) accounts for over 50% of all chronic liver disease in industrialized countries[1-3] and was responsible for more than 25,000 deaths in 2005 in the United States alone.[4] ALD comprises various degrees of liver injury ranging from alcoholic fatty liver (with or without inflammation) to alcohol-induced hepatic fibrosis and cirrhosis. Although nearly all heavy drinkers show signs of

Abbreviations: AC, alcoholic control; AFL, alcoholic fatty liver; ALC, alcoholic liver cirrhosis; ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CI, confidence interval; MAF, minor allele frequency; OR, odds ratio.

From the [1]Institute of Clinical Pharmacology and Visceral Research, University of Bern, Bern, Switzerland; the [2]Department of Internal Medicine I, [8]Department of General and Thoracic Surgery, and [17]Institute of Medical Informatics and Statistics, University Hospital Schleswig-Holstein, Kiel, Germany; the Departments of [3]Community Medicine, [4]Pharmacology, and [11]Gastroenterology, University Hospital Greifswald, Greifswald, Germany; the [5]Department of Gastroenterology, University Hospital Leipzig, Leipzig, Germany; the Departments of [6]Psychiatry and [15]Gastroenterology, University of Regensburg, Regensburg, Germany; the [7]Central Institute of Mental Health and [9]Salem Medical Center, University of Heidelberg, Mannheim, Germany; the [10]Psychiatric Hospital Meiringen, Meiringen, Switzerland; the [12]Department of Gastroenterology, University Hospital Homburg, Homburg, Germany; the [13]Department of Internal Medicine I, University Hospital Frankfurt, Frankfurt, Germany; the [14]Department of Gastroenterology, University Hospital Düsseldorf, Germany; and the [16]Department of Internal Medicine, General Hospital Oberndorf, Austria.

Received March 19, 2010; accepted September 18, 2010.

Supported by the German National Genome Research Network (NGFN) through the POPGEN biobank (BmBF 01GR0468) and NGFN plus (BmBF 01GS08152) and the BmBF systems biology networks QuantLiver (BmBF 0313853D) and Virtual Liver. The Community Medicine Research net (CMR) of the University of Greifswald is funded by the Federal Ministry of Education and Research, the Ministry of Cultural Affairs, and the Social Ministry of the Federal State of Mecklenburg-West Pomerania. The CMR encompasses several research projects sharing data from the population-based Study of Health in Pomerania.

**These authors contributed equally to this work.*

hepatic steatosis, only 10%-35% of alcoholic individuals develop hepatic inflammation and 10%-20% progress to cirrhosis.[5,6] Apart from the pattern and amount of alcohol consumption, coinfection with hepatitis B and C virus, female sex, obesity, and other features of the metabolic syndrome contribute to the development of alcoholic cirrhosis.[7,8] However, because these factors are not sufficient to explain the wide diversity of hepatic damage, a role of genetic factors is suspected. In fact, twin studies have suggested that approximately 50% of the phenotypic variation of alcoholism can be attributed to genetic factors.[9,10]

Recently, a genome-wide screen of nonsynonymous single-nucleotide polypmorphisms in a population of Hispanic, African, and European Americans from the Dallas Heart Study identified a strong association of a variant in *PNPLA3* (rs738409 [M148I]) with hepatic fat content as measured using proton magnetic resonance spectroscopy.[11] In the subgroup of Hispanic extraction, a similarly strong association with elevated alanine aminotransferase (ALT) levels was observed. The association between *PNPLA3* variants and ALT serum levels[12,13] and hepatic fat content were subsequently confirmed in subjects of Finnish,[14,15] Argentinian,[16] southern European,[17] and German[18] ancestry.

In addition, Tian et al.[19] investigated a possible relation with clinically evident liver disease using 17 variants in *PNPLA3* and a panel of 306 ancestry-informative single-nucleotide polymorphisms in a Mestizo (mixed European and Native American ancestry) population with a history of heavy alcohol abuse. A significant association of *PNPLA3* rs738409 with alcoholic cirrhosis (unadjusted odds ratio [OR] 2.25; $P = 1.7 \times 10^{-10}$) was found. Due to marked differences in allele frequencies of *PNPLA3* rs738409(G) across populations, an ancestry-adjusted analysis was performed that confirmed the initial observation (adjusted OR 1.79; $P = 1.9 \times 10^{-5}$). The difference of five orders of magnitude on the significance level due to ethnic substratification underscores the need to evaluate variant *PNPLA3* rs738409(G/C) in independent and potentially less genetically heterogenous samples. Analyses of regional or ethnic stratification in German Caucasians have not revealed significant evidence of genetic substrata,[20] thus suggesting that Germany is a suitable setting for such replication studies. We therefore investigated the impact of variant *PNPLA3* rs738409 on ALD in two independent German study populations.

Patients and Methods

Alcoholic Patients (Multicenter Sample). Alcoholic patients with a long-term history of chronic alcohol abuse were recruited from gastroenterology, psychiatry, and addiction medicine departments of the university hospitals of Regensburg, Mannheim, Heidelberg, Lübeck, Erlangen, Frankfurt, Kiel, and Homburg, all in Germany. Patient cohorts were reported previously,[21,22] and enrollment protocols were reported in detail both for aspects related to liver disease[21] and those related to psychiatry.[22] A recruitment algorithm depicting sample size, patient inclusion, exclusion, characterization, and group assignment is provided in Fig. 1. In brief, consecutive alcoholic patients for whom sufficient clinical data were available and who had past and/or present heavy alcohol consumption ($\geq$60 g/day for women and $\geq$80 g/day for men) for more than 10 years were recruited between 2000 and 2009 in the participating centers. To confirm the eligibility of patients, present alcohol consumption was quantified during a face-to-face interview. All patients from Regensburg and Mannheim received a diagnosis of alcohol dependence (per DSM-IV criteria) by the consensus of two clinical psychiatrists. All patients underwent careful clinical examination, standard laboratory testing, and abdominal ultrasound. Chronic viral hepatitis was excluded in all patients by testing for hepatitis B surface antigen, antibody to hepatitis B core antigen, and third-generation hepatitis C antibody enzyme-linked immunosorbent assay. Serum levels of ferritin and transferrin saturation were determined to rule out hereditary hemochromatosis, and neither clinical nor serological signs of autoimmune liver disease were present. All patients and controls were of self-reported German ancestry.

The presence of cirrhosis was determined on the results of a liver biopsy (fibrosis stage 4) or unequivocal clinical and laboratory evidence for the presence of cirrhosis as reflected by a combination of (1) standard liver blood tests (aminotransferases, gamma glutamyl transpeptidase, coagulation tests, serum albumin

Address reprint requests to: Jochen Hampe, Klinik für Innere Medizin I, Christian-Albrechts-Universität Kiel, Schittenhelmstr. 12, 24105 Kiel, Germany. E-mail: jhampe@1med.uni-kiel.de; fax: (49)-431-597-1842.

Copyright © 2010 by the American Association for the Study of Liver Diseases.

View this article online at wileyonlinelibrary.com.
DOI 10.1002/hep.24017
Potential conflict of interest: Dr. Wodarz serves on the speaker's bureau of Essex Pharmaceuticals and Jansen Cilag.
Additional Supporting Information may be found in the online version of this article.

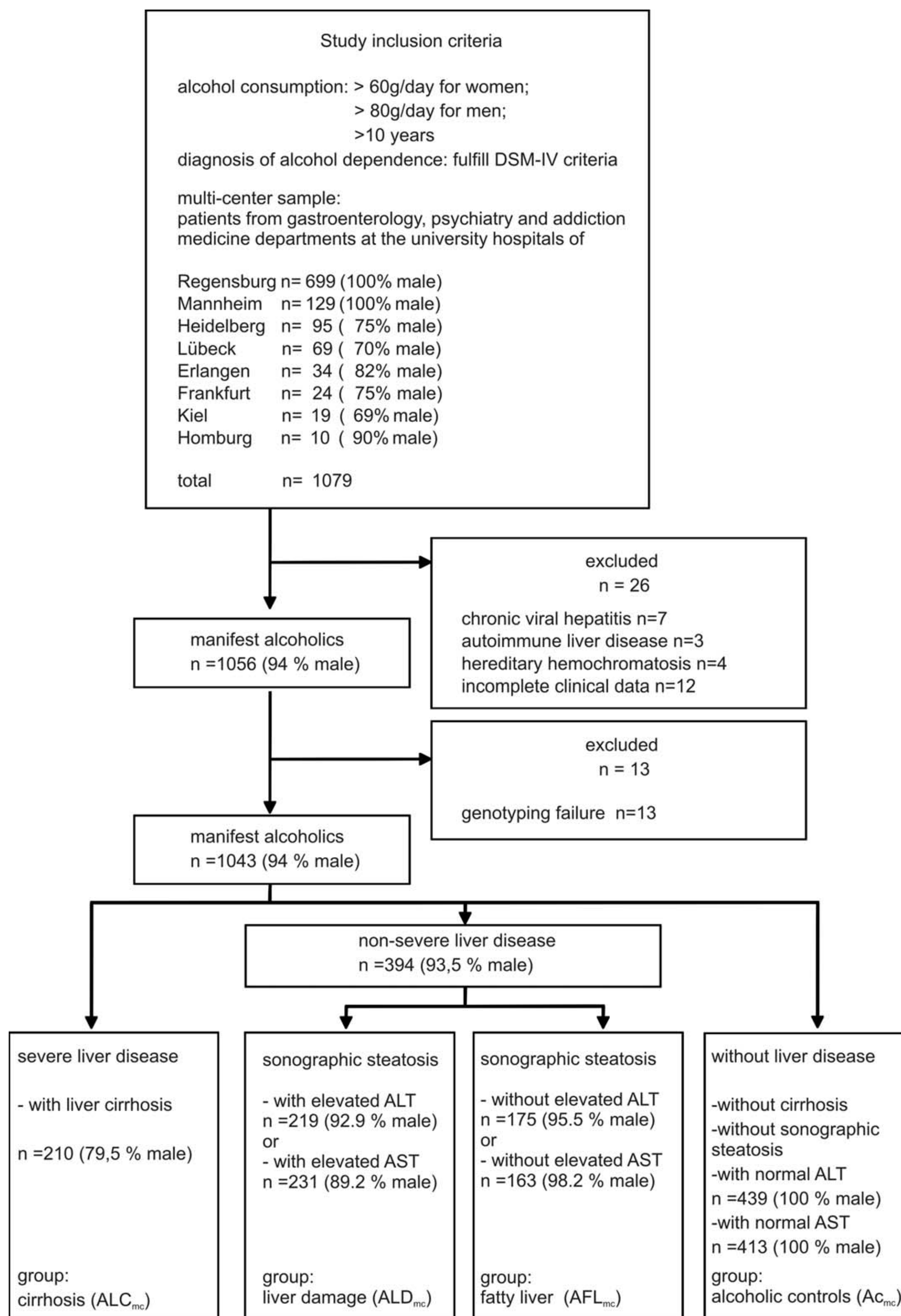

Fig. 1. Algorithm depicting the recruitment of alcoholic patients and controls for the multicenter sample, their characterization, and corresponding group assignment.

concentration, platelet count), (2) cirrhosis-related complications including encephalopathy or ascites, (3) sonographic and/or radiological (computed tomography) findings suggestive of cirrhosis (hunched liver surface, ascites, splenomegaly), and (4) detection of esophageal varices via upper gastrointestinal endoscopy. Liver biopsies were scored according to Ishak et al.[23] The presence of steatosis was assessed by increased echogenicity of liver parenchyma on abdominal ultrasound in relation to echogenicity of the kidney cortical

parenchyma at each individual center. ALT and aspartate aminotransferase (AST) levels were determined as part of routine clinical chemistry and scored as elevated based on the thresholds established by the laboratories at the individuals centers. Based on these phenotypic variables, patients were assigned to the following groups: (1) alcoholic liver cirrhosis (ALC), (2) alcoholics with liver steatosis on ultrasound and elevation of ALT (alcoholic liver damage [ALD]), (3) alcoholic liver steatosis and normal liver enzyme levels (alcoholic fatty liver [AFL]), and (4) alcoholic patients with normal appearance of the liver on ultrasound and normal liver enzyme levels (alcoholic controls [ACs]). In addition, similar assignments were made using AST. All assignments were made by the clinical investigators blinded to the genotype data.

At-Risk Drinkers (Population-Based Sample). Subjects from the Study of Health in Pomerania were recruited as described.[24] In brief, 4,310 adult German residents (20-79 years of age) of West Pomerania in Northeast Germany were recruited in between 1997 and 2001 and underwent an extensive multidimensional phenotyping, which has been analyzed and reported.[25-27] An algorithm depicting patient recruitment, characterization, and group assignment is shown in Fig. 2. Alcohol consumption was assessed using the beverage-specific quantity-frequency measure as described by Baumeister et al.[24]: number of days of alcohol consumption (beer, wine, spirits) and average daily alcohol consumption per day over the past month. Average daily consumption (in grams of pure ethanol per day) was calculated by multiplying frequency and amount using beverage-specific standard ethanol contents. Serum ALT levels were measured photometrically (Hitachi 704; Roche, Mannheim, Germany) and are expressed as μmol/L × second, which corresponds to (μmol/L × second) × 60 = IU/L. Serum ALT levels exceeding the upper reference limit recommended by the manufacturer (0.67 [40.2 IU/L] in men and 0.52 [31.2 IU/L] in women) were considered increased. Assessment of steatosis (presence or absence thereof) and subject grouping based on ultrasound findings, serum ALT levels and daily alcohol consumption resembled that of the multicenter sample and created four categories: (1) alcoholic subjects with steatosis and increased serum ALT levels (ALD), (2) alcoholics with sonographic evidence of fatty liver but normal serum ALT levels (AFL), (3) alcoholic subjects with normal liver echogenicity and ALT levels within the normal range (AC), and (4) nonalcoholic control subjects who were either abstainers or had a daily alcohol consumption of <10 g. All groups were matched for alcohol intake, sex, body mass index (BMI) and diabetes. In addition, groups were assigned using serum AST instead of ALT levels.

Genotyping. DNA from all samples was prepared using the FlexiGene chemistry (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA samples were evaluated by way of gel electrophoresis and adjusted to 20-30 ng/μL DNA content using the Picogreen fluorescent dye (Molecular Probes, Carlsbad, CA). One microliter of genomic DNA was amplified with the GenomiPhi (Amersham, Uppsala, Sweden) whole-genome amplification kit and fragmented at 99°C for 3 minutes. Five nanograms of DNA were dried overnight in TwinTec hardshell 384-well plates (Eppendorf, Hamburg, Germany) at room temperature. Genotyping rs738409 (hcv7241) was performed using the Taqman chemistry (Applied Biosystems, Foster City, CA) on an automated platform with Tecan Freedom EVO and 384-well TEMO liquid handling robots (Tecan, Männedorf, Switzerland). All process data were logged and administered using database-driven LIMS software.[28] Reactions were completed and read in a 7900 HT TaqMan sequence detector system (Applied Biosystems). The amplification reaction was performed with the TaqMan universal master mix. Thermal cycling conditions consisted of one cycle for 10 minutes at 95°C, 45 cycles for 15 seconds at 95°C, and 45 cycles for 1 minute at 60°C.

Data Analysis. The study was performed using a case-control design: Primary analyses were performed in patients with manifest alcohol abuse. The key inclusion criterion was defined alcohol dependence. No additional matching was performed as shown in Fig. 1 and as explained in the Alcoholics (Multicenter Sample) section in Patients and Methods. Confirmatory analyses were performed in a patient sample drawn from a population-based study. Matching was performed using the patients with alcoholic liver damage (ALD_p) as a reference. The other phenotypic categories were drawn randomly from the population study to provide matching of the median alcohol intake (identical medians), BMI (medians within 1%), sex (difference <5%), and diabetes status (differences <2.5%) to control for the main risk factors contributing to alcoholic liver damage and steatosis. Details are provided in Table 1, Fig. 2, and Supporting Information Table 2. Comparisons of frequencies of the three genotypes CC, CG, and GG were performed on a 3 × 2 contingency table between phenotypic groups. Herein, separate analyses based on serum ALT and AST levels were performed. For allelic tests, the frequency of the risk (G) allele was compared between groups, thereby

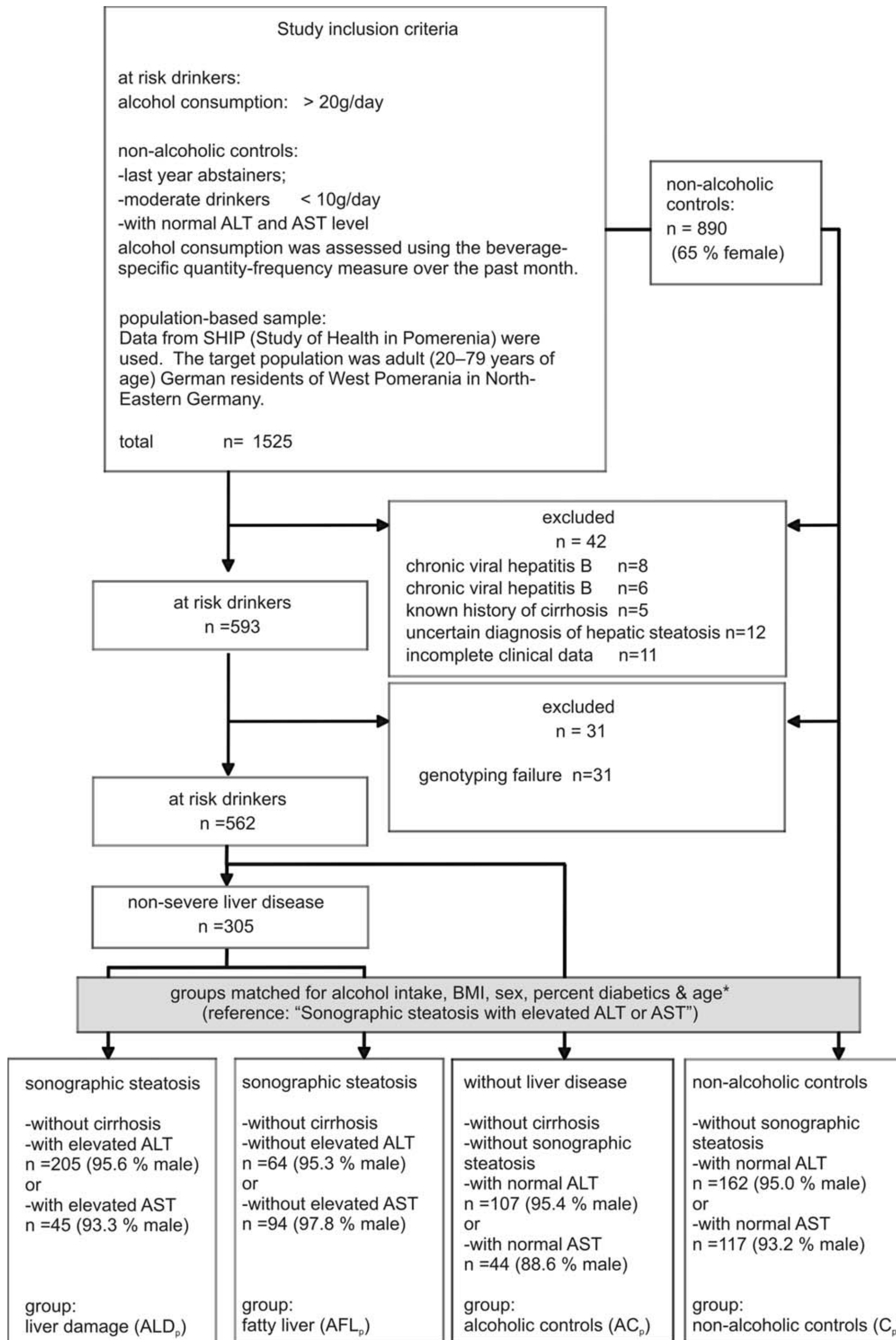

Fig. 2. Recruitment, phenotyping, and allocation to different study groups for the population-based sample. *Matching for the known risk factors of alcohol intake, BMI, sex, and diabetes status took precendence over age matching.

aggregating the CG genotypes (allele count G: 1) and GG genotypes (allele count G: 2). Genotypic and allelic tests of association genotype results were performed using χ^2 statistics or Fisher's exact tests on these contingency tables as appropriate. Nominal P values are reported for all tests. To determine the proportion of the excess phenotype manifestation that might be attributable to a specific genotype, the population attributable risk in percent (PAR%) was calculated as:

$$PAR\% = \frac{f_{GT}(RR-1)}{f_{GT}(RR-1)+1}\,100$$

where f_{gt} is the frequency of the genotype in the at-risk population and RR refers to the genotypic relative risk as estimated by the respective ORs. The population attributable risk or fraction provides an epidemiological estimate of which proportion of the disorder is

Table 1. Characteristics of Alcoholic Patients and Control Subjects

Group	n	ALT (IU/L)	AST (IU/L)	Age (Years)	Male Sex (%)	BMI (kg/m^2)	Diabetes (%)
			Multicenter sample				
Alcoholic liver cirrhosis (ALC_{mc})	210	25.5 (14-39.3)	39 (19.5-72.5)	50 (43-55)	79.5	25.4 (23.0-28.2)	ND
Alcoholic liver damage (ALD_{mc})	219	84 (66.3-134.8)	75 (51-124.3)	45 (38-54)	92.9	26.4 (24.2-30.4)	3
Alcoholic fatty liver (AFL_{mc})	175	25.5 (17-33)	22 (14-34.5)	43 (37-50)	95.5	24.3 (22.1-25.4)	4.5
Alcoholic controls (AC_{mc})	439	21 (15-29)	16 (11-25)	39 (34-45)	100	n/d	2.2
			Population-based sample				
Alcoholic liver damage (ALD_p)	205	55.2 (42.9-67.5)	33 (27.6-41.6)	48 (41-56)	95.6	29.5 (26.7-32.4)	5.6
Alcoholic fatty liver (AFL_p)	64	25.2 (21.3-29.1)	21.6 (19.6-24.7)	63 (58-68)	95.3	29.6 (27.2-32.1)	7.2
Alcoholic controls (AC_p)	107	23.9 (19.1-29.4)	20.4 (17.4-22.8)	55 (43-68)	94.4	29.3 (27.5-31.2)	4.7
Nonalcoholic controls (C_p)	162	21.3 (16.8-27.6)	19.2 (16.8-21.6)	66 (59-72)	95.0	29.1 (27.6-31.0)	5.0

ND, not determined.

Patients and controls were phenotyped as described in Patients and Methods. Group assignment of patients is based on ALT levels. Quantitative measures are summarized as median (25th percentile and 75% percentile [Q1-Q3]). Controls in the population-based sample were either abstainers or drank $<$10 g alcohol per day.

attributable to the risk factor (here, the genotype) in question. In other words, it provides an estimate on how much less alcoholic liver damage would be present in the population if the risk genotype would be eliminated from the population.

Results

Association with Manifestations of ALD in the Multicenter Sample. Details of patients and controls are provided in Table 1 and Supporting Information Tables 1 and 2. Analyses were first performed in the multicenter sample using the 439 alcoholic patients (AC_{mc}) without sonographic, clinical, or biochemical evidence of liver damage as a reference group for statistical tests. Allelic and genotypic association of rs738409 with AC (n = 210) was highly significant ($P_{allelic} = 1.6 \times 10^{-6}$; $P_{genotype} = 1.2 \times 10^{-5}$). The OR for homozygous carriers of the risk allele rs738409 was estimated as $OR_{ALCmc(GG)}$ 3.60 (95% confidence interval [CI] 1.95-6.64) compared with $OR_{ALCmc(CG)}$ 2.01 (95% CI 1.44-2.81) and $OR_{ALCmc(G)}$ 1.86 (95% CI 1.44-2.40) for carriership of the rs738409(G) allele. Association was also detected in comparison with patients ALD (ALD_{mc}, $P_{allelic} = 4.2 \times 10^{-3}$; $P_{genotype} = 8.5 \times 10^{-3}$) with corresponding ORs of $OR_{ALDmc(GG)}$ 2.55 (95% CI 1.37-4.73) for homozygotes compared with heterozygous carriers of the risk allele was observed ($OR_{ALDmc(CG)}$ 1.42 [95% CI 1.02-1.96]). Interestingly, in AFL patients, the G allele and GG genotype showed a tendency toward underrepresentation ($P_{genotype}$ = 0.11) with corresponding ORs of $OR_{AFLmc(GG)}$ 0.31 (95% CI 0.09-1.06) and $OR_{AFLmc(CG)}$ 0.77 (95% CI 0.53-1.11). The frequency of the risk allele rs738409(G) thus increases with increasing biochemical evidence of alcoholic hepatocyte damage as depicted in the upper panel of Fig. 1. A similar trend is evident for the homozygote rs738409(GG) genotype for steatosis with normal ALT ($f_{AFLmc(GG)}$ = 1.7%), no discernible changes in sonography and normal ALT ($f_{ACmc(GG)}$ = 5%), alcoholic liver damage and elevated ALT ($f_{ALDmc(GG)}$ = 11%), and cirrhosis ($f_{ALCmc(GG)}$ = 12.9%). Complete results of genotyping are provided in Table 2.

Because an AST/ALT ratio of >1 is a typical feature of ALD, and elevated AST levels are considered specific for alcohol-related liver injury, an analysis based on AST values was performed. Results are presented in Fig. 3A and in Supporting Information Tables 2 and 3. The results are indeed very similar to those from the ALT-based analysis.

Association with Manifestations of ALD in the Population-Based Sample. Alcohol intake and the prevalence of associated liver damage has been assessed systematically in the population-based Study of Health in Pomerania.[24] Using a cutoff of 140 g/week of alcohol intake as a threshold of at-risk exposure to alcohol, a group of 107 individuals (median group alcohol intake 300 g/week) without detectable liver damage was used as a reference for analysis. Using a phenotype of combined elevated ALT levels and increased echogenicity of the liver, significant association of rs738409(G) homozygosity with ALD was confirmed ($P_{allelic}$ = 0.024; $P_{genotype}$ = 0.04). A more pronounced risk was detected with homozygosity $OR_{ALDp(GG)}$ 5.32 (95% CI 1.19-23.77) and heterozygosity $OR_{ALDp(CG)}$ 1.53 (95% CI 0.95-2.47). Again, subjects with steatosis and liver enzyme levels within the normal range exhibited a trend toward minor allele frequency (MAF) of the rs738409(G) allele at 0.156 versus 0.196 for controls (genotypic P = 0.33; $OR_{AFLp\ (G)}$ 0.76 [95% CI 0.42-1.36] and $OR_{AFLp(CG)}$ = 0.66 [95% CI 0.34-1.28]). The allele frequencies are plotted against each of the three groups of alcoholic patients in the lower panel of Fig. 1. An analysis

Table 2. Genotype Frequencies and Association Analysis of Groups Based on Serum ALT Levels

Group	N	Genotype CC	Genotype CG	Genotype GG	MAF (%)	f_{CG} (%)	f_{GG} (%)	OR (95% CI) G versus C	OR (95% CI) GG+ GC versus CC	OR (95% CI) GG versus GC+CC	Comparison	$P_{allelic}$	$P_{genotype}$
								Multicenter sample					
Cirrhosis (ALC_{mc})	210	90	93	27	35.0	44.3	12.9	1.86 (1.44-2.40)	2.01 (1.44-2.81)	2.79 (1.55-5.04)	ALC_{mc} versus AC_{mc}	1.60×10^{-6}	1.18×10^{-5}
Alcoholic liver damage (ALD_a)	219	113	82	24	29.7	37.4	11.0	1.46 (1.13-1.89)	1.42 (1.02-1.96)		ALD versus AC	0.0042	0.0085
Liver damage (ALD_{mc})	219	113	82	24	29.7	37.4	11.0	1.46 (1.13-1.89)	1.42 (1.02-1.96)	2.33 (1.27-4.26)	ALD_{mc} versus AC_{mc}	0.0042	0.0085
Fatty liver (AFL_{mc})	175	116	56	3	17.7	32.0	1.7	0.74 (0.54-1.02)	0.77 (0.53-1.11)	0.33 (0.09-1.11)	AFL_{mc} versus AC_{mc}	0.067	0.111
								2.50 (1.78-3.51)	2.62 (1.73-3.97)	8.45 (2.52-28.4)	ALC_{mc} versus AFL_{mc}	7.84×10^{-8}	5.78×10^{-7}
Alcoholic controls (AC_{mc})	439	264	153	22	22.4	34.9	5.0						
								Population-based sample					
Liver damage (ALD_p)	205	107	81	17	28.0	39.5	8.3	1.60 (1.07-2.38)	1.53 (0.95-2.47)	4.75 (1.08-20.9)	ALD_p versus AC_p	0.021	0.040
Fatty liver (AFL_p)	64	46	16	2	15.6	25.0	3.1	0.76 (0.42-1.36)	0.66 (0.34-1.28)	1.69 (0.23-12.3)	AFL_p versus AC_p	0.352	0.334
Alcoholic controls (AC_p)	107	67	38	2	19.6	35.5	1.9						
Nonalcoholic controls (C_p)	162	104	55	3	18.8	34.0	1.9	0.95 (0.61-1.47)	0.93 (0.56-1.55)	0.99 (0.16-6.02)	C_p versus AC_p	0.817	0.965
								AFL (combined *post hoc* analysis)					
Fatty liver (ALF_{mc} + ALF_p)	239	162	72	5	17.2	30.1	2.1	0.74 (0.56-0.98)	0.73 (0.53-1.01)	0.46 (0.17-1.23)	AFL_{tot} versus AC_{tot}	0.032	0.086
Alcoholic controls (AC_{mc}+ AC_p)	546	331	191	24	21.9	35.0	4.4						

MAF, minor allele frequency.

Results of the genetic association analysis of coding single-nucleotide polymorphism rs738409 (M148I) in the *PNPLA3* gene are shown, including genotype counts, genotype frequencies, and minor allele frequencies. *P* values are reported for allelic ($P_{allelic}$) and genotypic association ($P_{genotype}$) tests.

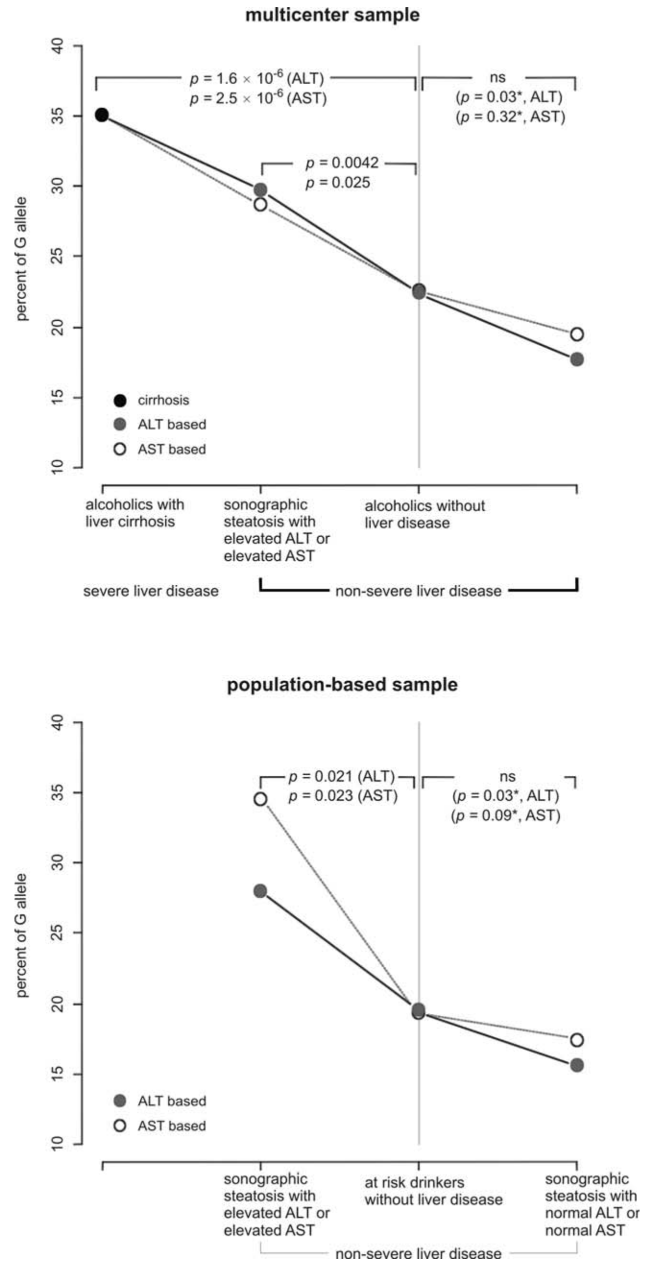

Fig. 3. Frequency of risk allele rs738409(G) in the clinical categories of alcohol-induced liver damage. (A) Results from the multicenter population of clinical alcoholics. (B) Results of the population-based study. Allelic *P* values for group comparisons are also given. The *P* value of the combined comparison of the AFL versus AC groups is noted in parentheses and is marked with an asterisk. The bracket below the *x* axis indicates the combined analysis in nonsevere liver disease in the multicenter sample (OR 1.11 [CI 0.89-1.40], $P = 0.353$ for ALT-based analysis and OR 1.11 [0.88-1.39], $P = 0.381$ for AST-based analysis) and population-based sample in the lower panel (OR 1.37 [CI 0.93-2.03], $P = 0.111$ for ALT-based analysis and OR 1.25 [CI 0.69-2.27], $P = 0.466$ for AST-based analysis).

based on AST concentrations was performed that revealed similar findings as in the multicenter sample. Detailed findings are reported in Fig. 3B and in Supporting Information Tables 2 and 3.

Because BMI was available for all subjects from the population-based sample, ALT values in different genotypes were analyzed with and without correction for BMI. As demonstrated in Supporting Information Fig. 1, BMI had no significant impact on serum ALT concentrations.

Combined Analysis of Steatosis. Combined analysis of subjects with steatosis and liver enzyme levels within the normal range compared with individuals without sonographic steatosis and normal ALT levels from both the multicenter and population-based study revealed a significantly lower frequency of the rs738409(G) allele at $MAF_{AFL} = 0.021$ versus $MAF_{AC} = 0.044$ (Fig. 1, Table 2).

Population-Attributable Risks for Alcoholic Liver Damage in Alcoholic Patients. The genotype allele frequencies from the population based control study of abstainers were located between those of AC and AFL ($P > 0.05$). Thus, the population-attributable risks for the development of alcoholic cirrhosis can be estimated at 4.6% (OR 3.6 [CI 1.95-6.64]) for homozygotes and 26.6% (OR 2 [CI 1.44-2.81]) for G allele carriership.

Discussion

This study demonstrates an association of alcoholic liver disease with the allele *PNPLA3* rs738409(G) in Caucasian alcoholic patients with a P value of ($P_{allelic} = 1.6 \times 10^{-6}$) for cirrhosis and 0.0042 for elevation of ALT. The latter finding was confirmed with a P value of 0.02 in a population-based sample of drinkers with a median alcohol intake of 300 g/week. No difference in genotype frequencies between ACs and healthy control subjects from the population-based study was observed rendering an association of the studied polymorphism, with alcohol intake itself unlikely. These results were confirmed in an AST-based analysis indicating a robust genotypic effect.

Our data underscore the significance of genotype *PNPLA3* rs738409(GG) in ALD recently demonstrated for an admixed population of Mestizo individuals.[19] The degree of admixture in the former study is reflected by five orders of magnitude of difference between the uncorrected P value ($P = 1.7 \times 10^{-10}$) and the ethnicity-adjusted significance level of ($P = 1.9 \times 10^{-5}$). This also emphasizes the important differences in population frequency of variants at PNPLA3, which may partly explain prevalence differences of alcoholic and nonalcoholic liver disease.[11] Based on systematic studies on evidence of population substructure,[20,29] German Caucasians do not exhibit a significant degree of substratification and can thus be grouped on the basis of phenotype alone as performed in previous studies.[25] Because no correction for ethnic background was necessary, a direct estimation of risk and ORs for different manifestations of ALD was possible. The genotypic OR of 3.6 for homozygous carriage of the risk genotype rs738409(GG) for cirrhosis is remarkably high for a complex trait such as alcoholic cirrhosis. This calculated risk for rs738409(GG) carriers is surprisingly consistent with the data of Tian et al.,[19] which can be estimated at $(1.81)^2 = 3.27$, confirming the accuracy of the ancestry correction in their study. The inclusion of the population-based cohort confirms the impact of the risk genotype for alcoholic liver damage even on a moderate level of risk intake of a median of 300 grams of alcohol per week.

Although genetic substratification is likely not a methodological problem of this study, the multicenter study comprises subjects diagnosed at many centers across Germany. Thus, differences in phenotypic assignment among centers and investigators cannot be fully ruled out. To specifically control for potential misclassification bias, an additional, population-based study with highly standardized phenotyping was included as an independent confirmation cohort.[24] Herewith, the ORs are similar to those calculated for patients in the multicenter study, including the underrepresentation of the risk genotype rs738409(GG) in heavy drinkers with ALT values within the reference range. Thus, phenotypic heterogeneity does not seem to preclude the conclusions derived from the multicenter study of clinical alcoholics.

Previous genetic reports have associated the variant both with hepatic fat content[11,14-18] and with markers of hepatocyte damage as reflected by elevated serum ALT and AST levels.[12,13] In both of our populations, the association of genotype rs738409(GG) with liver cirrhosis and ALT elevation is consistent. Whether this association depends on variable degrees of steatosis cannot be answered with these data, because limited information from only a subset of patients with available liver histologies prevent sufficiently powered analyses. Apart from the initial ALT-based analysis, we have also analyzed the complete dataset with an AST-based approach paying tribute to the frequent elevation of AST over ALT levels in alcoholics, which confirmed the association of genotype rs738409(GG) with AST elevation underlining a robust biological basis for the observed genotypic effects. However, the power of the AST-based analyses seemed somewhat lower than in the ALT approach. We have no precise explanation for the lower specificity of AST elevation to detect liver damage in these individuals, but a confounding effect from extrahepatic sources of AST might contribute to

this observation. Obesity, however, does not seem to add to the association of genotype rs738409(GG) with ALD.

It remains an unresolved issue why carriage of the rs738409(G) allele increases the susceptibility to develop alcoholic liver damage and liver enzyme elevations, whereas carriage of the C allele confers protection. What seems to be a stable observation in both of our populations is the low frequency of the risk allele rs738409(G) in drinkers with sonographic steatosis and no biochemical evidence of relevant alcoholic liver injury, suggesting protection from progression to more advanced alcoholic liver damage when the risk variant is absent. This finding suggests a mechanism leading merely to steatosis, and a second molecular link promoting necroinflammatory activity driven by the rs738409(G) genotype. Along this line, we hope our report inspires other investigators to reach a mechanistic understanding of these processes. Clearly, sonographic echogenicity can serve only as an approximation of the presence or absence of liver steatosis. Thus, this finding needs to be further investigated in patient samples with biopsy-based assessment of steatosis, inflammation (apoptosis), and fibrosis.

The functional implications of PNPLA3 and its genetic variation are still incompletely defined and currently subject to intense research. PNPLA3, also referred to as adiponutrin, encodes a 481–amino acid protein that belongs to the patatin-like phospholipase domain-containing family.[30] Human PNPLA3 is closely related to adipose triglyceride lipase PNPLA2, a major triglyceride hydrolase of adipose tissue.[31,32] Recently, He et al.[33] reported a functional study of the allelic effects of PNPLA3 rs738409(G) on triglyceride metabolism and subcellular localization in cellular and mouse model systems. Their data are compatible with PNPLA3 rs738409(G) primarily promoting triglyceride accumulation by limiting triglyceride hydrolysis by demonstrating that the wild-type enzyme hydrolyzes emulsified triglycerides, whereas the variant offsets this activity. Expression of PNPLA3 rs738409(G), but not of wild-type PNPLA3 rs738409(C), increased cellular triglyceride content in cultured hepatocytes or in the livers of mice.

In the past two decades, the search for genetic factors that predispose carriers to ALD has resulted in a large number of hypothesis-driven candidate gene case-control studies. Herein, associations between alcoholic liver injury with single-nucleotide polymorphisms within genes coding for alcohol-metabolizing enzymes,[34] cytokines, antioxidant stress, and others were reported.[35] In spite of this abundance of data and effort, confirmation of any of the genetic variants as risk factors for ALD in independent cohorts is still lacking. In this respect, great expectations came with the advent of genome-wide association studies. In fact, recent genome-wide association studies have identified a large number of robust associations between specific chromosomal loci and complex human disease, including liver diseases.[11,25,36] Our present study exploits this innovation by evaluating a genetic variant previously identified as a risk factor through genome-wide testing (*PNPLA3* and nonalcoholic fatty liver disease) through its testing in another type of liver disease (alcoholic liver disease).

We conclude that *PNPLA3* rs738409(G/G) carriers represent a genetically defined subpopulation of high-risk individuals susceptible to progression of clinically inapparent to overt alcoholic liver disease. Indeed, considering these two extremes of the spectrum of alcoholic liver injury are considered, an OR of 11.6 (CI 3.41-39.46) is obtained. In total, 26.6% of the population attributable risk for the progression of early to advanced alcoholic liver disease are conferred by the presence of this risk allele. Homozygous carriers of genotype rs738409(GG) should thus be considered a target group for future rigorous pharmacological and nonpharmacological interventions.

References

1. Corrao G, Ferrari P, Zambon A, Torchio P, Arico S, Decarli A. Trends of liver cirrhosis mortality in Europe, 1970-1989: age-period-cohort analysis and changing alcohol consumption. Int J Epidemiol 1997;26:100-109.
2. Kim WR, Brown RS Jr, Terrault NA, El-Serag H. Burden of liver disease in the United States: summary of a workshop. HEPATOLOGY 2002; 36:227-242.
3. John U, Hanke M. Alcohol-attributable mortality in a high per capita consumption country—Germany. Alcohol Alcohol 2002;37:581-585.
4. Yoon YH, Yi HY. Surveilance report #83: liver cirrhosis mortality in the United States, 1970-2005. Arlington, VA: National Institute on Alcohol Abuse and Alcoholism; 2008.
5. Teli MR, Day CP, Burt AD, Bennett MK, James OF. Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver. Lancet 1995;346:987-990.
6. Teli MR, James OF, Burt AD, Bennett MK, Day CP. The natural history of nonalcoholic fatty liver: a follow-up study. HEPATOLOGY 1995; 22:1714-1719.
7. Bellentani S, Saccoccio G, Costa G, Tiribelli C, Manenti F, Sodde M, et al. Drinking habits as cofactors of risk for alcohol induced liver damage. The Dionysos Study Group. Gut 1997;41:845-850.
8. Raynard B, Balian A, Fallik D, Capron F, Bedossa P, Chaput JC, et al. Risk factors of fibrosis in alcohol-induced liver disease. HEPATOLOGY 2002;35:635-638.
9. Reed T, Page WF, Viken RJ, Christian JC. Genetic predisposition to organ-specific endpoints of alcoholism. Alcohol Clin Exp Res 1996;20: 1528-1533.
10. Hrubec Z, Omenn GS. Evidence of genetic predisposition to alcoholic cirrhosis and psychosis: twin concordances for alcoholism and its biological end points by zygosity among male veterans. Alcohol Clin Exp Res 1981;5:207-215.
11. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet 2008;40:1461-1465.

12. Yuan X, Waterworth D, Perry JR, Lim N, Song K, Chambers JC, et al. Population-based genome-wide association studies reveal six loci influencing plasma levels of liver enzymes. Am J Hum Genet 2008;83:520-528.
13. Kollerits B, Coassin S, Kiechl S, Hunt SC, Paulweber B, Willeit J, et al. A common variant in the adiponutrin gene influences liver enzyme levels. J Med Genet 2010;47:116-119.
14. Kotronen A, Johansson LE, Johansson LM, Roos C, Westerbacka J, Hamsten A, et al. A common variant in PNPLA3, which encodes adiponutrin, is associated with liver fat content in humans. Diabetologia 2009;52:1056-1060.
15. Kotronen A, Peltonen M, Hakkarainen A, Sevastianova K, Bergholm R, Johansson LM, et al. Prediction of non-alcoholic fatty liver disease and liver fat using metabolic and genetic factors. Gastroenterology 2009;137:865-872.
16. Sookoian S, Castano GO, Burgueno AL, Fernandez Gianotti T, Rosselli MS, Pirola CJ. A nonsynonymous gene variant in adiponutrin gene is associated with nonalcoholic fatty liver disease severity. J Lipid Res 2009;50:2111-2116.
17. Romeo S, Sentinelli F, Dash S, Yeo GS, Savage DB, Leonetti F, et al. Morbid obesity exposes the association between PNPLA3 I148M (rs738409) and indices of hepatic injury in individuals of European descent. Int J Obes (Lond) 2010;34:190-194.
18. Kantartzis K, Peter A, Machicao F, Machann J, Wagner S, Konigsrainer I, et al. Dissociation between fatty liver and insulin resistance in humans carrying a variant of the patatin-like phospholipase 3 gene. Diabetes 2009;58:2616-2623.
19. Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in PNPLA3 is associated with alcoholic liver disease. Nat Genet 2010;42:21-23.
20. Steffens M, Lamina C, Illig T, Bettecken T, Vogler R, Entz P, et al. SNP-based analysis of genetic substructure in the German population. Hum Hered 2006;62:20-29.
21. Stickel F, Osterreicher CH, Halangk J, Berg T, Homann N, Hellerbrand C, et al. No role of matrixmetalloproteinase-3 genetic promoter polymorphism 1171 as a risk factor for cirrhosis in alcoholic liver disease. Alcohol Clin Exp Res 2008;32:959-965.
22. Treutlein J, Cichon S, Ridinger M, Wodarz N, Soyka M, Zill P, et al. Genome-wide association study of alcohol dependence. Arch Gen Psychiatry 2009;66:773-784.
23. Ishak KG, Zimmerman HJ, Ray MB. Alcoholic liver disease: pathologic, pathogenetic and clinical aspects. Alcohol Clin Exp Res 1991;15:45-66.
24. Baumeister SE, Volzke H, Marschall P, John U, Schmidt CO, Flessa S, et al. Impact of fatty liver disease on health care utilization and costs in a general population: a 5-year observation. Gastroenterology 2008;134:85-94.
25. Buch S, Schafmayer C, Volzke H, Becker C, Franke A, von Eller-Eberstein H, et al. A genome-wide association scan identifies the hepatic cholesterol transporter ABCG8 as a susceptibility factor for human gallstone disease. Nat Genet 2007;39:995-999.
26. Volzke H, Schwarz S, Baumeister SE, Wallaschofski H, Schwahn C, Grabe HJ, et al. Menopausal status and hepatic steatosis in a general female population. Gut 2007;56:594-595.
27. Wolff B, Grabe HJ, Volzke H, Ludemann J, Kessler C, Dahm JB, et al. Relation between psychological strain and carotid atherosclerosis in a general population. Heart 2005;91:460-464.
28. Hampe J, Wollstein A, Lu T, Frevel HJ, Will M, Manaster C, et al. An integrated system for high throughput TaqMan based SNP genotyping. Bioinformatics 2001;17:654-655.
29. Nelis M, Esko T, Magi R, Zimprich F, Zimprich A, Toncheva D, et al. Genetic structure of Europeans: a view from the North-East. PLoS One 2009;4:e5472.
30. Baulande S, Lasnier F, Lucas M, Pairault J. Adiponutrin, a transmembrane protein corresponding to a novel dietary- and obesity-linked mRNA specifically expressed in the adipose lineage. J Biol Chem 2001;276:33336-33344.
31. Kienesberger PC, Oberer M, Lass A, Zechner R. Mammalian patatin domain containing proteins: a family with diverse lipolytic activities involved in multiple biological functions. J Lipid Res 2009;50(Suppl.):S63-S68.
32. Wilson PA, Gardner SD, Lambie NM, Commans SA, Crowther DJ. Characterization of the human patatin-like phospholipase family. J Lipid Res 2006;47:1940-1949.
33. He S, McPhaul C, Li JZ, Garuti R, Kinch LN, Grishin NV, et al. A sequence variation (I148M) in PNPlA3 associated with nonalcoholic fatty liver disease disrupts triglyceride hydrolysis. J Biol Chem 2010;285:6706-6715.
34. Zintzaras. Do alcohol-metabolizing enzyme gene polymorphisms increase the risk of alcoholism and alcoholic liver disease? HEPATOLOGY 2006;43:352-361.
35. Stickel F, Osterreicher CH. The role of genetic polymorphisms in alcoholic liver disease. Alcohol Alcohol 2006;41:209-224.
36. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.